The conserved actinobacterial two-component system MtrAB coordinates chloramphenicol production with sporulation in *Streptomyces venezuelae* NRRL B-65442

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Abstract. *Streptomyces* bacteria make numerous secondary metabolites, including half of all known antibiotics. Production of antibiotics is usually coordinated with the onset of sporulation but the cross regulation of these processes is not fully understood. This is important because most *Streptomyces* antibiotics are produced at low levels or not at all under laboratory conditions and this makes large scale production of these compounds very challenging. Here we characterise the highly conserved actinobacterial two-component system MtrAB in the model organism *Streptomyces venezuelae* and provide evidence that it coordinates production of the antibiotic chloramphenicol with sporulation. MtrAB are known to coordinate DNA replication and cell division in *Mycobacterium tuberculosis* where TB-MtrA is essential for viability but MtrB is dispensable. We deleted *mtrB* in *S. venezuelae* and this resulted in a global shift in the metabolome, including constitutive, high-level production of chloramphenicol. We found that chloramphenicol is detectable in the wild-type strain, but only at very low levels and only after it has sporulated. ChIP-seq showed that MtrA binds upstream of DNA replication and cell division genes and genes required for chloramphenicol production. *dnaA*, *dnaN*, *oriC* and *wblE* (*whiB1*) are DNA binding targets for MtrA in both *M. tuberculosis* and *S. venezuelae*. Intriguingly, over-expression of TB-MtrA and gain of function TB- and Sv-MtrA proteins in *S. venezuelae* also switched on high level production of chloramphenicol. Given the conservation of MtrAB, these constructs might be useful tools for manipulating antibiotic production in other filamentous actinomycetes.

Introduction.

Streptomyces secondary metabolites account for two thirds of all known antibiotics and numerous other compounds that are used in human medicine as anticancer, anti-parasitic, antiviral and immunosuppressant drugs (Devine et al., 2017). Discovery of these natural products peaked in the 1950s but there has been a resurgence of interest in the 21st century, driven by genome sequencing and the increasing threat of drug resistant infections (Katz and Baltz, 2016). Despite their importance however, we still have a poor understanding of how *Streptomyces* bacteria control the production of their secondary metabolites. This is important because ≥75% of their secondary metabolite biosynthetic gene clusters (BGCs) are not expressed in laboratory culture and activating cryptic BGCs could facilitate the discovery of new antibiotics and other useful natural products (Hosaka et al., 2009; Rutledge and Challis, 2015; Antoraz at al., 2015).

The major way in which bacteria sense and respond to their environment is through two-component systems and several have been implicated in the regulation of antibiotic production in *Streptomyces* species (Hsiao and Kirby, 2009; Rodriguez et al., 2013). Two component systems typically consist of a bifunctional sensor kinase and a cognate response regulator (Salazar and Laub, 2015). The sensor kinase perceives an extracellular signal and activates its cognate response regulator through a two-step phosphorylation. The phosphorylated regulator (RR~P) brings about a response to the original signal, usually by modulating target gene expression. In the absence of signal, the bifunctional sensor kinases dephosphorylate their cognate regulators to keep the response switched off (Capra and Laub, 2012). The delicate balance of kinase and phosphatase activities is crucial in modulating the activity of the response regulator and its target genes during the bacterial life cycle (Salazar and Laub, 2015). Cross-talk between two component systems is rare in wild-type cells but removal of a sensor kinase can result in constitutive activation of its response regulator by a non-cognate sensor kinase or the cellular pool of acetyl phosphate (Capra and Laub, 2012; Hutchings, 2007). Similarly, altering a sensor kinase to block its phosphatase activity can result in a response regulator that cannot be dephosphorylated and is rendered constitutively active (Salazar and Laub, 2015).

Here we report characterisation of the highly conserved actinobacterial two component system MtrAB in the model organism *Streptomyces venezuelae* NRRL B-65442 (Hoskisson and Hutchings, 2006). MtrA was first identified as an essential regulator in *Mycobacterium tuberculosis* (Mycobacterium tuberculosis regulator A) (Zahrt and Deretic, 2000). *M. tuberculosis* MtrA (TB-MtrA) regulates the expression of *dnaA* and *dnaN*, which are essential for DNA replication, and sequesters the origin of DNA replication, *oriC*, in dividing cells. TB-MtrA also regulates the expression of cell division genes and interacts directly with the DnaA protein to prevent the initiation of DNA replication (Purushotham et al., 2015). The TB-MtrB sensor kinase is activated following localisation to the site of cell division through interaction with FtsI and DivIVA (Wag31) and these data have led to a model in which oscillations in TB-MtrA~P levels play a key role in cell cycle progression with TB-MtrA~P repressing DNA replication and activating cell division (Plocinska et al., 2012; 2014; Purushotham et al., 2015). An accessory lipoprotein called LpqB modulates the activity of MtrB in mycobacteria and an *M. smegmatis* Δ*lpqB* mutant grows as *Streptomyces*-like filaments (Nguyen et al., 2010). In the closely related *Corynebacterium glutamicum*, Cg-MtrA is not essential and controls genes

involved in cell wall remodelling and the osmotic stress response (Brocker et al., 2011; Krämer, 2009) but deletion of the *mtrAB* genes gives rise to elongated cells which are indicative of a defect in cell division (Möker et al., 2004).

Streptomyces bacteria are filamentous saprophytes which grow through the soil as branching substrate mycelium that extends at the hyphal tip (Bush et al., 2015)s. Nutrient starvation triggers the production of reproductive aerial hyphae that accelerate DNA replication, generating up to 200 copies of the chromosome in each aerial hypha, before undergoing cell division to form chains of unigenomic spores (Bush et al., 2015). Aerial hyphae production and sporulation are coordinated with the production of antibiotics. *S. venezuelae* NRRL B-65442 has recently emerged as a good model for studying development because it completes a full developmental life cycle in liquid growth medium in just 20 hours (Munnoch et al., 2016). Here we present evidence that MtrAB coordinates chloramphenicol production with sporulation in *S. venezuelae*. ChIP-seq shows that MtrA binds directly to genes required for chloramphenicol production and deletion of *mtrB* resulted in a global shift in the metabolome and constitutive, high level production of chloramphenicol. MtrA also shares DNA binding targets with *M. tuberculosis* MtrA (*dnaA*, *dnaN*, *oriC* and *wblE/whiB1*) and binds to additional cell division genes including *ssgA*, *ssgB* and *ftsZ*. Expression of TB-MtrA and gain of function TB-MtrA and Sv-MtrA proteins in *S. venezuelae* also activates chloramphenicol production and we propose that manipulating MtrA activity could be a way of upregulating antibiotic production in other *Streptomyces* species.

MATERIALS AND METHODS

Strains, plasmids and primers. The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. Liquid cultures of *E. coli* were routinely grown shaking at 220 rpm, in Lennox Broth (10g tryptone, 5g yeast extract, 5g NaCl made up to 1L with deionised water) at 37°C. Liquid cultures of *S. venezuelae* NRRL B-65442 were grown in Mannitol Yeast Extract Malt Extract (MYM; Kieser et al., 2000) at 30°C, shaking at 220 rpm with springs. Cultures grown on solid MYM agar were grown at 30°C. To determine the growth rate of *S. venezuelae* strains in liquid culture, a spore inoculum sufficient to reach an OD_{600} of 0.35 after 8 hours of growth was added to 35ml of MYM media in 250 ml flasks containing springs and grown at 30°C at 220rpm. Absorbance was measured at OD_{600} every two hours for 20 hours and samples were examined with a GXML3000B microscope from GX optical to determine developmental growth phase. Antibacterial bioassays were performed on MYM agar. Mutants were made using the Redirect PCR targeting protocol (Gust et al., 2003) and genes were introduced *in trans* using the integrative vectors pIJ10770 or pIJ10257 (Table S1). All strains and plasmids used in this study are either commercially available or available on request. Plasmids were introduced into *S. venezuelae* by conjugation with *E. coli* ET12567/pUZ8002 (Table S1) as described (Kieser et al., 2000).

Immunoblotting. Sv-MtrA-6His was overexpressed in *E. coli* BL21 using the pETDuet vector and purified on an AKTA FPLC system using a 5mL Hitrap nickel affinity column (GE Healthcare). Polyclonal antiserum was raised against purified Sv-MtrA-6His protein in rabbits by Cambridge Research Biochemicals. Immunoblotting was carried out as described previously (Hutchings et al., 2006b).

ChIP-seq. Chromatin ImmunoPrecipitation followed by sequencing (ChIP-seq) was performed on *S. venezuelae* NS003 (Δ*mtrA* ΦBT1 *mtrAp mtrA*-3xFlag; Supplementary Table 1) grown with springs in liquid MYM with wild-type *S. venezuelae* NRRL B-65442 as a control. This strain undergoes a full life cycle in 20 hours under these conditions. Samples were taken at 8, 10, 12, 14, 16, 18 and 20 hours. ChIP-seq was performed using anti-Flag antibodies (Sigma-Aldrich) and analysed as described previously (Bush et al., 2013; Crack et al., 2015; Munnoch et al., 2016). Peaks were visually inspected using integrated genome browser (Nicol et al., 2009).

Method development and validation for chloramphenicol quantitation. To quantify chloramphenicol, we developed a method based on a high-performance liquid chromatography (HPLC) 1100 system (Agilent Technologies) coupled with a photodiode array detector (DAD). The analysis was performed using a Gemini® 3 µm NX-C18 110 Å at ambient temperature and a flow rate of 0.8 mL/min. We applied a gradient of water/0.1% formic acid/methanol, starting conditions: 90/10, to 0/100 within 14 minutes, hold for 4 minutes, to 90/10 within 0.5 minutes, hold for 1 minute. We validated the method as per the International Conference on Harmonization (ICH) guidelines in terms of specificity, sensitivity, linearity, precision, accuracy and robustness (Supplementary Tables 2 and 3).

Sample preparation and analysis. *S. venezuelae* wild-type, Δ*mtrB*, and *ermEp*-mtrA* strains were grown in biological triplicates and technical triplicates in MYM medium. An aliquot of 750 µL was taken from each culture and 750 µL of ethyl acetate was added. After shaking for 10 minutes, the extracts were centrifuged and the organic phase transferred to a 1.5 mL glass vial. The solution was concentrated under reduced pressure and re-dissolved in 500 µL of a mixture of methanol/water (2:3). For quantification 50 µL of each sample was subjected to analytical HPLC. Stock solutions of chloramphenicol for calibration were prepared between a range of 0.01 $\mu g.mL^{-1}$ and 0.05 $mg.mL^{-1}$. The stock solutions were extracted and the calibration references prepared in the same way as the actual samples. Generation of the calibration curve and quantification of chloramphenicol was accomplished by integration of the chloramphenicol signal at 273 nm appearing between 10.99 and 11.05 minutes.

Method validation.

Sensitivity - and limit of quantification (LOQ)

The Limit of detection (LOD) describes the lowest amount of an analyte in a sample that can be detected but not necessarily quantified. The Limit of quantification (LOQ) is the lowest amount of an analyte in a sample that can be determined with suitable precision and accuracy. LOD for the method described here was calculated based on multiplying the noise level by 3 and was found to be 0.008 µg/mL. The LOQ was calculated by multiplying the noise level by 10 and was found to be 0.026 µg/mL. The noise level was determined by measuring a blank sample of methanol. LOD and LOQ were experimentally verified.

Linearity and calibration curve

Linearity of the method was evaluated by creating a calibration curve as a function of the concentration of the standard analyte chloramphenicol in µg/ml (X) and its peak area (Y) in the

HPLC trace at 273 nm. We found calibration curves to be linear over a concentration range of 0.01 µg/mL – 2 µg/mL. The calibration curve gave a good linear regression (Y= 164.454X-0.0001701, r^2 = 0.9998).

Precision and accuracy

We found the developed method to be precise as the relative standard deviation (RSD) values for repeatability of intra-day and inter-day precision studies were below the limit of 2.5% recommended by the ICH guidelines[10]. To determine the level of accuracy we performed a recovery study by adding three different amounts of a standard to a blank sample. The overall recovery percentages were in the range of 103.2% - 108.5% which demonstrates a decent accuracy for the quantitation of chloramphenicol.

Robustness

Robustness is defined as a measure of the method's capability to remain unaffected by small, but deliberate variations in some of the methods parameters. We introduced small changes in the HPLC method which may affect the reliability of the method such as flow rate, organic content in mobile phase and wavelength of detection. We observed a low overall RSD between data of varied conditions indicating a good robustness of the method.

Metabolomics analysis.

We cultivated three individually isolated Δ*mtrB* mutants and the wild-type in five biological replicates in MYM liquid medium for three days. Each sample was analysed in technical replicates. An aliquot of 750 µL was taken from each culture and 500 µL of methanol was added. The mixture was shaken for 10 minutes, centrifuged and 200 µL of the extract transferred to a 1.5 mL glass vial with a 250 µL glass insert. UPLC-HRMS Data was acquired on an Acquity UPLC system (Waters Corporation) equipped with an ACQUITY UPLC® BEH 1.7 µm C18, 1.0 × 100 mm column (Waters Corporation) and connected to a Synapt G2-Si high resolution mass spectrometer (Waters Corporation). A gradient of mobile phase A (H_2O with 0.1 % formic acid) and mobile phase B (acetonitrile with 0.1 % formic acid) at a flow rate of 80 µL/min was used. Initial conditions were 1 % B for 1 minute, ramped to 90 % B within 6 minutes, ramped to 100 % B within 0.5 minute, held for 0.5 minutes, returned to 1 % B within 0.1 minute and held for 1.9 minutes. MS spectra were acquired with a scan time of one second in the range of m/z = 150 - 1200 in positive MSe-Resolution mode. The following instrument parameters were used: capillary voltage of 3 kV, sampling Cone 40, source offset 80, source temperature 120 °C, desolvation temperature 350 °C, desolvation gas flow 800 L/h. We used a solution of sodium formate for calibration. A solution of leucine encephalin (H_2O/MeOH/formic acid: 49.95/49.95/0.1) was used as lock mass and was injected every 15 seconds during LCMS runs. The lock mass has been acquired with a scan time of 0.3 seconds and three scans were averaged for each timepoint. The lock mass (m/z = 556.2766) has been directly applied during data acquisition. All solvents for analytical HPLC and UPLC-MS were obtained commercially at least in HPLC grade from Fisher Scientific and were filtered prior to use. Formic acid (0.1 %) was added to the water. We used the Software Progenesis QI (Waters) for processing metabolomics data. The t-test ($p < 0.05$) and fold change analysis (fold change > 2) were applied to identify statistically significant differences between the control group (wild-type) and the Δ*mtrB* mutant. EZInfo 3.0 (MKS Umetrics AB) was used for plotting PCA data.

RESULTS AND DISCUSSION

Deleting *mtrB* switches on antibiotic production in *S. venezuelae* NRRL B65442. To investigate the function of the MtrAB two component system in *S. venezuelae* we attempted to delete the *mtrA* and *mtrB* genes using a PCR targeting approach (Gust et al., 2003). *mtrB* was deleted but all attempts to delete *mtrA* were unsuccessful until we introduced a second copy of *mtrA in trans.* This suggests MtrA might be essential in this strain but given that an *mtrA* mutant has been reported in *Streptomyces coelicolor* this will require further verification (Clark et al., 2013). Deletion of *mtrB* had no effect on growth rate in liquid culture (Figure 1A) but immunoblotting with polyclonal MtrA antibodies against wild-type *S. venezuelae* and the Δ*mtrB* mutant suggests that MtrA is over-produced in the absence of MtrB and to the same levels as when *mtrA* is expressed from the high level, constitutive *ermE** promoter (Figure 1B). Phenotypic screening of the Δ*mtrB* mutant also revealed that it inhibits the growth of *Bacillus subtilis* whereas neither the wild-type strain or the wild-type expressing *ermEp*-mtrA* has antibacterial activity (Figure 1B). The simplest explanation of these data is that deletion of *mtrB* results in constitutive phosphorylation of MtrA and that MtrA~P then constitutively activates expression of its target genes. We previously reported a similar result for the *S. coelicolor* response regulator VanR which is constitutively active in the absence of its cognate sensor kinase, VanS (Hutchings et al., 2006a). VanR and MtrA both belong to the OmpR family of response regulators and activation of the DNA binding activity in OmpR family proteins by phosphorylation is well established (Friedland et al., 2007).

The *S. venezuelae* Δ*mtrB* strain constitutively produces chloramphenicol. We reasoned that the antibacterial activity switched on in the *mtrB* mutant is most likely due to the production of chloramphenicol, which is reported to be switched off in wild-type *S. venezuelae* NRRL B-65442 (Fernández-Martínez et al., 2014). To further investigate this, we measured production of chloramphenicol in biological and technical triplicates of the wild-type strain and the Δ*mtrB* mutant after 12, 16, 24 and 36 hours of growth in liquid MYM medium (Figure 2). Consistent with the link between antibiotic production and sporulation in streptomycetes, we could detect low levels of chloramphenicol in cultures of the wild-type strain but only after it had sporulated (Figure 2). Cultivation for 24 hours in biological and technical triplicates confirmed an increased production of chloramphenicol in the Δ*mtrB* mutant with a mean concentration of 0.41 mg/L which is >30 times higher than the wild-type strain (0.013 mg/L) or wild-type over-expressing *mtrA* (0.010 mg/L). Importantly, chloramphenicol production is constitutive in the *S. venezuelae* Δ*mtrB* strain. Chloramphenicol biosynthesis is presumably switched on much earlier in the growth cycle of the Δ*mtrB* strain because we detect the antibiotic in actively growing cultures (12h) and cultures that are differentiating into aerial hyphae and spores (16-36h, Figures 1 and 2).

Deletion of *mtrB* results in a global shift in the metabolome. To examine the effects of deleting *mtrB* on the metabolome of *S. venezuelae*, we cultivated the wild-type strain and three independently isolated *ΔmtrB* mutants in biological and technical triplicates and analysed the extracts by UPLC/HRMS using untargeted metabolomics. Runs were aligned to compensate for between-run variation and a peak-picking algorithm was applied to allow for the

immaculate matching of each feature (a discrete *m*/*z* value and its retention time) among all runs. Following normalisation, features could be compared quantitatively and their putative identity proposed based on their high-resolution MS-signal. Comparing the level of metabolite signals, it appeared that all three *ΔmtrB* mutants showed an extensive alteration of global metabolite levels. To display multidimensional data, we used Principle Component Analysis (Figure 3). Each sphere in the 3D Plot represents one dataset obtained from a single UPLC-HRMS run. Data from the individual *ΔmtrB* mutant strains clearly group together, and are distinct from data obtained from the wild-type, while variations within each group are comparably small. The 3D Plot therefore shows consistent and global changes in the metabolome upon loss of MtrB (Figure 3).

MtrA binds directly to genes required for chloramphenicol production. Most sensor kinases are bifunctional and act as phosphatases in the absence of signal. This prevents aberrant activation of their response regulators through cross-talk. Removal of a sensor kinase can lead to activation of a response regulator by either the cellular pool of acetyl phosphate or by non-cognate sensor kinases. We hypothesise that loss of MtrB results in constitutive phosphorylation of MtrA and activation of its target genes and it seemed likely that these targets include genes in the chloramphenicol BGC. To test this, we performed ChIP-seq against a strain expressing a C-terminally 3xFlag-tagged MtrA protein from its native promoter, integrated at the ϕBT1 site, with the native *mtrA* gene deleted. The fact we could delete *mtrA* in a strain carrying this construct suggested that MtrA-3xFlag is functional and the strain grew like the wild-type (Supplementary Figure 1). We performed ChIP-seq on cultures grown for 8, 10, 12, 14, 16, 18 and 20 hours in liquid MYM medium (NCBI Geo database accession number GSE84311; Figure 1; Som et al., 2016). Reads were mapped to the *S. venezuelae* NRRL B-65442 genome (Genbank accession number CP018074) and the chloramphenicol BGC was examined for MtrA ChIP peaks. The data show significant enrichment (cut-off $P < 0.05$) between the divergent *cmlN* and *cmlF* genes in the chloramphenicol BGC, which encode transporters required for chloramphenicol production (Fernández-Martínez et al., 2014). This indicates direct binding by MtrA (Figure 4A). MtrA also binds between the divergent *jadR1* and *jadR2* genes in the jadomycin BGC and this may affect chloramphenicol production. JadR1 activates and JadR2 represses jadomycin biosynthesis but they also cross regulate the chloramphenicol BGC in other *S. venezuelae* strains (Zhang et al., 2013; Forget et al., 2017). JadR1 represses expression of the chloramphenicol BGC and is itself repressed by JadR2 (Liu et al., 2013) so MtrA could feasibly activate JadR2 and / or repress JadR1 since it is known to act as both activator and repressor in other actinomycetes. However, we do not yet know if MtrA regulates expression of these genes. Under the conditions used we did not detect any jadomycins in the wild-type or Δ*mtrB* strains using LCMS with verified jadomycin standards. However, the phenotype of the Δ*mtrB* mutant suggests that MtrB is required to coordinate chloramphenicol production with sporulation in *S. venezuelae*, presumably through phosphorylation / dephosphorylation of MtrA. Consistent with this we found that, as in *M. tuberculosis*, *S. venezuelae* MtrA binds to essential targets involved in DNA replication and cell division (Figure 4B and Table 1). The MtrA targets shared between *M. tuberculosis* and *S. venezuelae* are *oriC*, *dnaA*, *dnaN* and *wblE*/*whiB1* (Table 1)(Purushotham et al., 2015). *oriC* is the origin of DNA replication in bacteria and is bound by multiple DnaA molecules which

melt the DNA to initiate DNA replication. In actinomycetes *oriC* falls in the intergenic region between *dnaN* and *dnaA*. *M. tuberculosis* MtrA sequesters *oriC* by binding to three sites and also represses expression of *dnaA* and *dnaN* {Purushotham:2015iq}. The *wblE* gene encodes a member of WhiB-like (Wbl) family of transcription factors, which is restricted to actinomycetes. They are not well understood but they appear to all contain 4Fe-4S iron sulphur clusters and nitrosylation of this cluster in the *M. tuberculosis* WblE homologue, WhiB1, activates the protein for DNA binding {Stapleton:2012ks}. The complete *M. tuberculosis* MtrA regulon has not been published so more targets may be conserved. MtrA likely autoregulates its own expression because there is an MtrA ChIP peak centred at -50 relative to the major transcription start site at +1, as determined by examining published differential RNA sequencing data (dRNA-seq; Figure S2) (Munnoch et al., 2016). The position of this MtrA binding site is consistent with auto-activation and could explain why the MtrA protein appears to be over-produced in the Δ*mtrB* strain (Figure 1). A second, minor transcript (P2) initiates upstream of the MtrA binding site at -79bp (Figures 5 and S2). Published dRNA-seq data for *S. coelicolor* also shows that the *mtrAB-lpqB* gene are expressed on a leaderless transcript starting at +1 relative to the *mtrA* translation start codon (Jeong et al., 2016). However, in *S. coelicolor* another gene, encoding the putative eukaryotic translation initiation factor Sco3014, has inserted immediately upstream of *mtrA* and P2 starts at +1 relative to the *sco3014* translation start codon (Figure 5).

Gene name	Gene number	Function	Enrichment value (≥7 is equivalent to P≤0,05 and considered statistically significant)					
			10	12	14	16	18	20h
	SVEN15_4502	GGDEF domain protein	8	4.8	1.6	0.3	0.8	20
cdgB	SVEN15_3942	GGDEF domain protein	4.8	3.5	1.5	0.4	0.4	10.7
	SVEN15_0422	GGDEF / EAL domain protein	2.2	3	2.2	1.2	1.2	3.8
rmdB	SVEN15_5058	GGDEF / EAL domain protein	5.6	2.9	0.6	0	0.5	18.9
	SVEN15_5080	GGDEF / EAL domain protein	3.8	2.8	1.7	0.8	0.8	4.3
bldG	SVEN15_3247	Anti-anti sigma factor	4.3	3.8	1.5			9.6
adpA / bldH	SVEN15_2524	Regulator	2.4	3.5	1.6	0.5	0.9	5.8
bldM	SVEN15_4355	Atypical RR	3.6	2.7	1.3	0.4	0.9	9.1
bldN	SVEN15_3116	ECF sigma factor	2.9	2.1	0.6	0.1	1.1	12.3
whiB	SVEN15_2717	WhiB-like (Wbl) protein	3.2	2.5	1	0.3	0.9	10.6
whiD	SVEN15_4354	WhiB-like (Wbl) protein	3.8	2.7	1.3	0.4	0.9	10
wblE	SVEN15_4802	WhiB-like (Wbl) protein	2.9	3.7	1.4	0.3	1.7	9.2
whiG	SVEN15_5191	Sigma factor	5.2	3.4	1.6	0.9	1.1	5
whiH	SVEN15_5388	GntR-like transcription factor	3.3	2.9	1.3	0.2	1	12
whiI	SVEN15_5706	Two-component RR	8.4	5.6	2.7	0.8	1.3	20.6
ssgA	SVEN15_3615	Regulator	1.3	1	0.4		0.9	8.3
ssgB	SVEN15_1102	Regulator	5.1	3.3	1.9	1.2	1.2	11.3
ssgD	SVEN15_6261	Regulator	2.6	2.8	1.3	0.6	0.4	2.8
ssgE	SVEN15_2937	Regulator	4.9	3.5	1.2	0.3	0.5	15.3
ssgG	SVEN15_2622	Regulator	8.7	4.7	1.7	0.5	0.9	6.4
chpH	SVEN15_1232		3.4	1.6	0.5		0.6	8.6

chpE	SVEN15_1396	Hydrophobic cell wall-associated proteins	0.4	0.8	0.6		0.3	1.4
chpG	SVEN15_4550		2.3	2.8	1	1.8	1.2	5.6
chpF	SVEN15_4546		2.8	2.3	2	1	1.6	7.8
chpD	SVEN15_4533		3.2	2.7	1	0.5	1.1	7.5
sapB	SVEN15_6339	Lantibiotic-like peptide surfactant	6.8	5.2	2.3	1	0.7	8.1
rdlA	SVEN15_4528	Secreted cell wall-associated protein	0.2	0.6	0.6	0.3	0.5	1
rdlB	SVEN15_4531		1.8	1.2	0.9	0.7	0.4	1.5
rdlA	SVEN15_4532		1.4	1.4	0.7	0.4	0.7	2.9
oriC	SVEN15_3570	origin of DNA replication	3.1	1.5	0.4		0.6	6.3
			1.2	2.3		0.1	0.7	8
dnaA	SVEN15_3571	Chromosome replication initiation protein	0.7	0.9				4.5
ftsZ	SVEN15_1692	Tubulin-like cell division protein	4.8	4	1.5	0.5	0.6	15.5
divIVA	SVEN15_1687	Tip-associated protein	1	2	1	0.5	0.5	8
filP	SVEN15_4942	Filament protein	3.2	2.4	1	0.4	0.5	9.8
smeA	SVEN15_0971	Small membrane protein	5.8	4.9	1.6	0.6	1.7	21.5
smc	SVEN15_5163	Chromosome-associated ATPase	3.2	2.5	1.1	0.9	0.9	2.4
mtrA	SVEN15_2696	Response regulator	10	7.7	2.4	1.2	1.4	19.4

Table 1. ChIP-seq data against MtrA-3xFlag. Enrichment values are given for target genes known or predicted to be involved in development. An enrichment value < 7 (P<0.05) was not considered statistically significant in this experiment.

***M. tuberculosis* MtrA switches on chloramphenicol production in *S. venezuelae*.** Sv-MtrA shares 75% primary sequence identity and several target genes with TB-MtrA so we were curious to see if expression of TB-MtrA or the gain-of-function Y102C TB-MtrA in *S. venezuelae* would permit deletion of *mtrA* at the native locus. We also modelled Sv-MtrA onto the published TB-MtrA structure using HHrep (Soding et al., 2006; Friedland et al., 2007) and made the equivalent Y99C (predicted gain-of-function) change in Sv-MtrA (Figure 6). We made expression constructs for all three, driven by the *S. venezuelae mtrA* promoter, and introduced them into the ϕBT1 phage integration site (Gregory et al., 2003). All three strains grew normally but we could not delete the native *mtrA* gene. Nevertheless, we measured chloramphenicol production in these strains and found that they all produced significantly more chloramphenicol than the wild-type (Figure 6) or wild-type over-expressing *mtrA* from the *ermE** promoter (see above). It is puzzling that wild-type TB-MtrA has such a large effect and

344 our only explanation is that TB-MtrA may be constitutively phosphorylated in *S. venezuelae*
345 and not subject to control by Sv-MtrB.
346
347 **CONCLUDING REMARKS.**
348 In this study, we have carried out the first characterisation of MtrAB in the genus *Streptomyces*,
349 using the model organism *S. venezuelae* NRRL B65542. ChIP-seq every two hours throughout
350 the life cycle indicate that the DNA binding activity of Sv-MtrA is developmentally regulated
351 and this, combined with the fact that Sv-MtrA binds to many developmental genes (Table 1)
352 suggests MtrAB plays a role in regulating life cycle progression in *S. venezuelae*. MtrA activity
353 changes during the life cycle and appears to be biphasic, being most active in vegetatively
354 growing substrate hyphae and in spores but less active in aerial hyphae that are undergoing
355 rapid DNA replication. This is most likely because MtrA is subject to developmental regulation
356 via phosphorylation / dephosphorylation by MtrB so it will be important to discover how MtrB
357 activity is regulated, e.g. by interaction with the cell division machinery as in *M. tuberculosis*
358 (Plocinska et al., 2012; 2014). One key role uncovered here for MtrAB is the coordination of
359 chloramphenicol production with sporulation. We have shown that deletion of *mtrB* uncouples
360 these processes and results in constitutive production of this antibiotic. It is perhaps surprising
361 that we do not see any effects on sporulation in the Δ*mtrB* strain but the developmental genes
362 bound by Sv-MtrA are subject to complex regulation which presumably cannot be over-ridden
363 simply by activating MtrA (Bush et al., 2015). Nevertheless, we have shown that manipulating
364 the activity of MtrAB proteins can be used to upregulate antibiotic production and given their
365 conservation in filamentous actinomycetes this may provide another useful tool to switch on
366 cryptic BGCs. Future work will focus on testing whether MtrA is essential, if and how it
367 regulates the gene targets identified using ChIP-seq and understanding how MtrB activity is
368 regulated in *Streptomyces* species.
369
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Figure Legends

Figure 1. Deletion of *mtrB* has no effect on growth rate but switches on antibacterial activity in *S. venezuelae* NRRL B-65442. A. Growth curves in liquid MYM medium for the wild-type strain (blue), isogenic Δ*mtrB* mutant (red) and the Δ*mtrB* mutant with *mtrB*

505 introduced *in trans* at the ϕBT1 phage integration site under the control of the *mtrAB-lpqB*
506 operon promoter (green). The phases of the life cycle are indicated and the coloured arrows
507 show the time points at which samples were taken for ChIP-seq experiments. B. Top panel
508 shows an immunoblot using polyclonal antiserum raised against purified MtrA. Samples were
509 analysed at 20h cultures. The lanes contain equal concentrations of total protein from whole
510 cell extracts from the wild-type *S. venezuelae* NRRL B-65442 strain, the isogenic Δ*mtrB*
511 mutant and the wild-type expressing *mtrA* from the high level, constitutive *ermE** promoter as
512 verified by Bio-Rad protein assay and Coomassie stained SDS PAGE gels (not shown). Bottom
513 panel. Bioassays of the same strains against *Bacillus subtilis* (Bsu) show that only the *S.*
514 *venezuelae* (Sven) Δ*mtrB* mutant has antibacterial activity.
515
516 **Figure 2. Chloramphenicol is produced constitutively and at higher levels in the absence**
517 **of MtrB.** Levels of chloramphenicol detected in wild-type *S. venezuelae* NRRL B-65442 (blue)
518 and the isogenic Δ*mtrB* mutant (red). Note that chloramphenicol is only produced following
519 sporulation in the wild-type but is produced at all stages of growth and at higher levels in the
520 Δ*mtrB* mutant. All measurements were on biological and technical triplicate samples.
521
522 **Figure 3. Loss of MtrB results in a global shift in the *S. venezuelae* NRRL B-65442**
523 **metabolome.** Principle Component Analysis on the wild-type (blue dots) and triplicate
524 samples of the *ΔmtrB* mutant (red, green and orange dots). Data from *ΔmtrB* mutant strains
525 clearly group together, and are distinct from data obtained from the wild-type while
526 variations within each group are comparably small.
527
528 **Figure 4A. MtrA binds upstream of genes affecting chloramphenicol production.** The
529 coloured lines show ChIP-seq data for MtrA-3xFlag at 8 hours (yellow), 10 hours (light
530 green), 12 hours (dark green), 14 hours (light blue), 16 hours (dark blue), 18 hours (purple)
531 and 20 hours (pink), see figure 1 for life cycle stages. The black line is the wild-type
532 (negative) control. ChIP peaks are shown at the divergent *cmlF* and *cmlN* genes, which
533 encode transporters required for chloramphenicol production (Fernández-Martínez et al.,
534 2014), and *jadR1* and *jadR2* genes, which encode transcription factors that cross-regulate the
535 jadomycin and chloramphenicol biosynthetic gene clusters (Liu et al. 2013).
536
537 **Figure 4B MtrA binds upstream of genes required for DNA replication and cell**
538 **division.** ChIP-seq peaks, as in Fig. 4, upstream of the DNA replication genes *dnaN* and
539 *dnaA* and the cell division genes *ftsZ* and *ssgB*. In actinomycetes the origin of DNA
540 replication, *oriC*, is between the *dnaN* and *dnaA* genes.
541
542 **Figure 5. Arrangement of the *mtrAB-lpqB* operon in *S. venezuelae* NRRL B-65442 and**
543 ***S. coelicolor* M145.** Published dRNA-seq data (Jeong et al., 2016; Munnoch et al., 2016)
544 shows that the major transcriptional start site is at +1 in both species, relative to the *mtrA*
545 translational start codon. A second transcript starts at -79 in *S. venezuelae* and an MtrA ChIP
546 peak is centred between P1 and P2 at -50, suggesting autoregulation. In *S. coelicolor* a gene
547 encoding a putative eukaryotic translation initiation factor has inserted immediately upstream
548 of *mtrA* and is also expressed on a leaderless transcript.

Figure 6. Activating chloramphenicol production using TB- and Sv-MtrA expression constructs. Top: Modelling *S. venezuelae* MtrA (Sv-MtrA) on the published *M. tuberculosis* MtrA (TB-MtrA) structure (Friedland et al., 2007) suggests they are likely to be highly similar. Bottom: Expression of TB-MtrA and the gain of function Y102C TB-MtrA proteins in wild-type *S. venezuelae* NRRL B-65442 switches on high level production of chloramphenicol. The equivalent Y99C change in Sv-MtrA has a smaller effect on chloramphenicol production.

Figure 1.TIFF

Figure 1

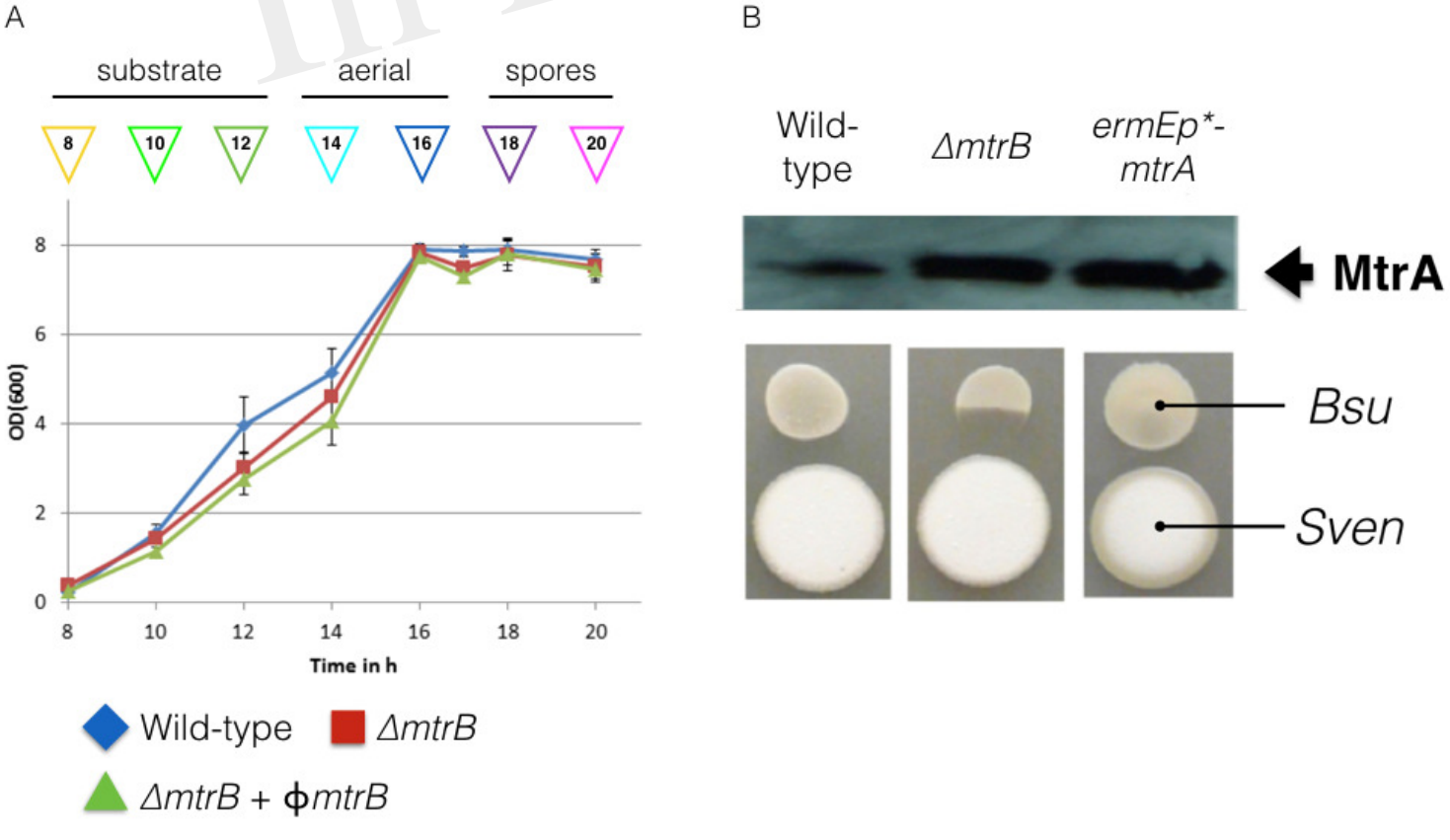

Figure 2.TIFF

Figure 2

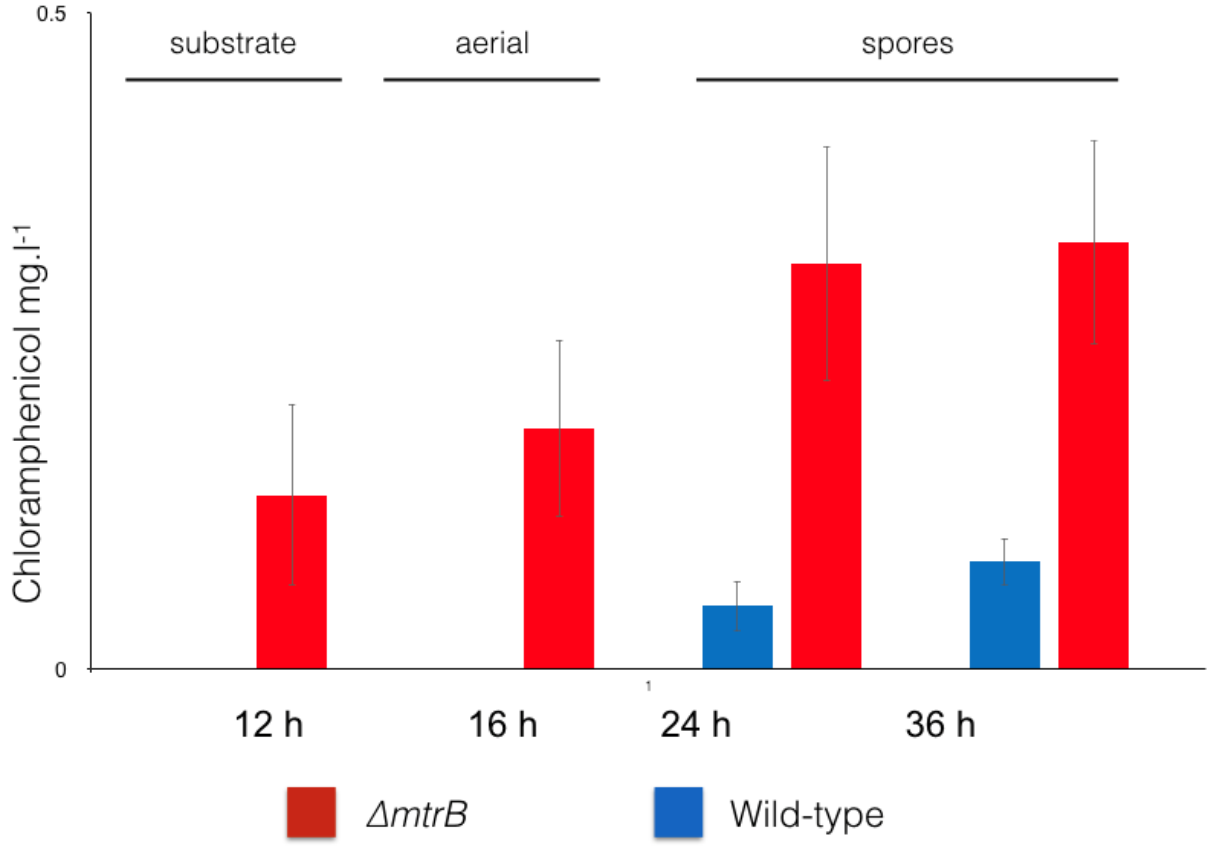

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Figure 3

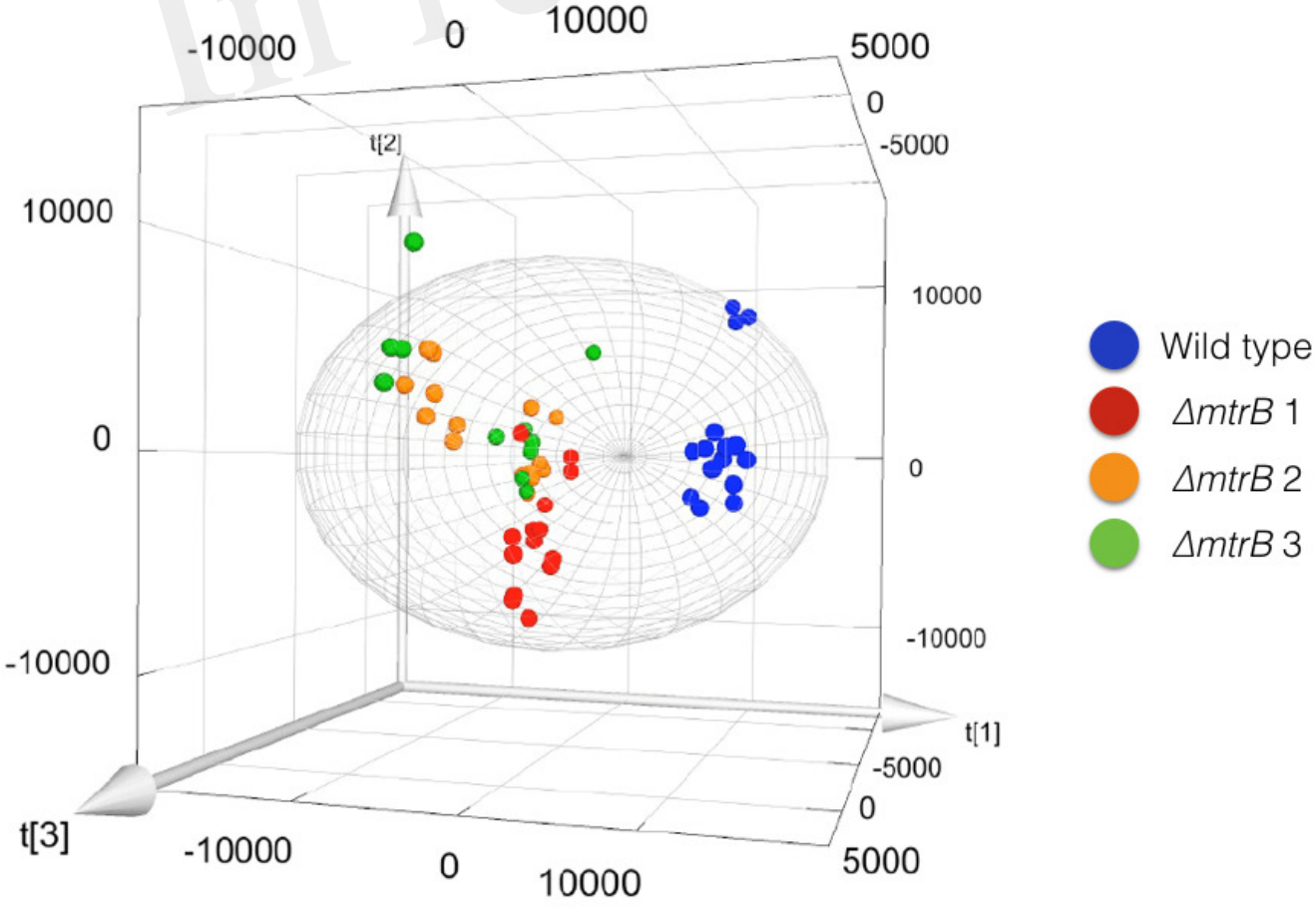

Figure 4

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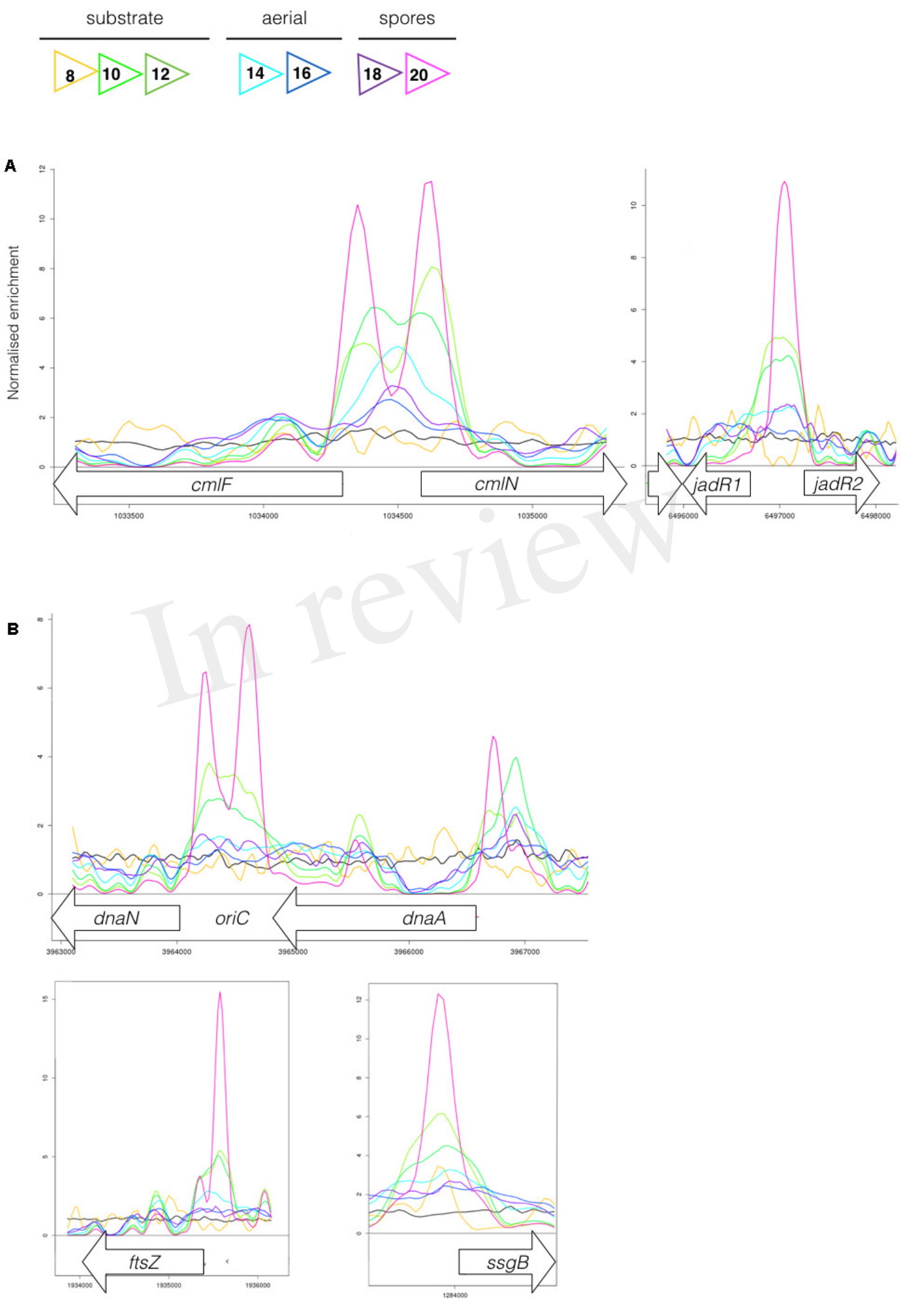

Figure 5.TIFF

Figure 5

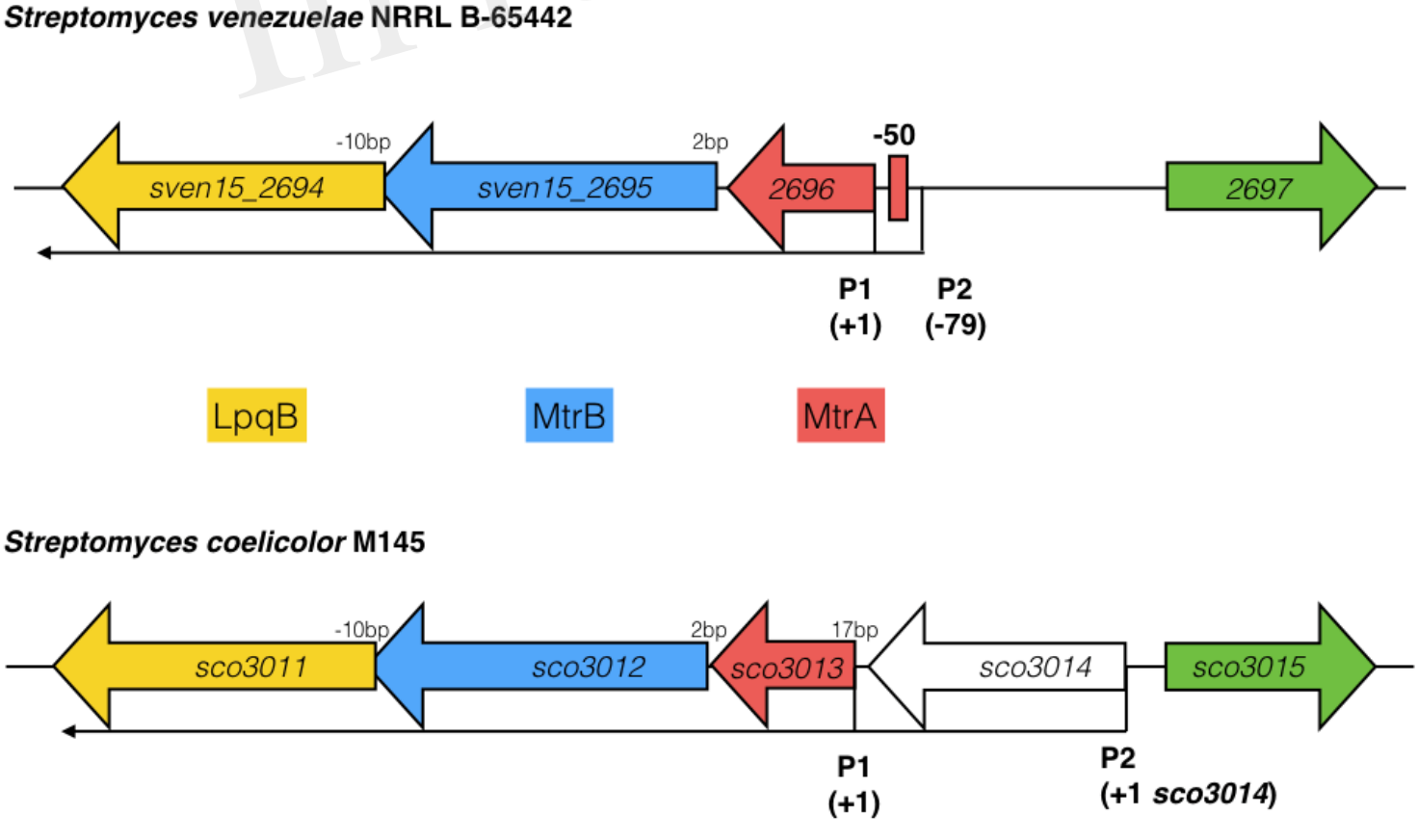

Figure 6.TIFF

Figure 6